REMARKS

Claims 29-35 are presently pending. Claim 29 is amended herein. Basis for the amendments may be found throughout the specification and claims as-filed, including in claim 1 as filed and at page 4, lines 4-7 and 17-24 of the specification. Claim 35 is cancelled without prejudice or disclaimer. No new matter is presented herein.

Rejections under 35 U.S.C § 112, second paragraph

Claims 29-35 are rejected under 35 U.S.C § 112, second paragraph a purportedly indefinite. Without acquiescing in the rejection, independent claim 29 is amended herein as suggested by the Office. Applicants request that this rejection be withdrawn.

Rejections under 35 U.S.C § 102(e)

Claims 29 and 31 stand rejected 35 U.S.C. § 102(e) as purportedly anticipated by Michael Gruenberg (US 2003/0175272 A1). Applicants respectfully submit that the '272 publication is not a proper reference under 35 U.S.C. § 102(e).

US Publication No. 2003/0175272, as cited herein under 35 U.S.C. 102(e), was published September 18, 2003 and was first filed anywhere on March 7, 2002. However, while the present application has a US national stage filing date of October 20, 2003 but claims priority back to DE 101 20 505.8, filed on April 26, 2001. The Office has acknowledged this priority claim on the record (see, for example, Office Action of December 3, 2007, Summary Page).

As the '272 publication here is asserted as prior art under 102(e), the filing date is considered the priority date, and so the 102(e) date of the '272 publication is March 7, 2002.

Thus, Applicants submit that the citation of the '272 publication is rebuttable based on the perfection of the priority claim of the present application to the April 26, 2001 filing date of DE 101 20 505.8.

Rejections under 35 U.S.C § 103(a)

Claims 29-32, 34 and 35 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Teruaki Sekine (0 409 655) in view of Flyer et al. (WO 97/32970). Claim 35 has been cancelled herein without prejudice or disclaimer. With regard to the application of this rejection to claims 29-32 and 34, Applicants respectfully submit that the method of amended independent claim 29 would not have been obvious to one of skill in the art in view of the combination of Sekine with Flyer.

Sekine discloses a multi-step method of stimulating the proliferation of peripheral blood lymphocytes (PBL) and their activation and/or differentiation into at least one cellular subpopulation consisting of helper T ($CD4^+$) cells, killer/suppressor T cells ($CD8^+$) or natural killer (NK) cells, in order to obtain a proliferated cellular population in a quantity suitable for use in adoptive immunotherapy (*see* abstract, page 3, lines 5-7, page 3, lines 22-25, and page 3, lines 40-43). The multi-step method of Sekine comprises contacting a small amount of starting peripheral blood lymphocytes with an anti-CD3 antibody immobilized to a culture substrate (*e.g.* an untreated culture flask), followed by a second step where the obtained cell population is transferred to a second uncoated culturing substrate (*e.g.* an uncoated culture flask) and allowed to continue culturing. A third step then takes place where the obtained proliferated cell population is transferred to a cell culturing substrate (*e.g.* a gas permeable cell culture bag) and contacted with a lymphokine (*e.g.* TNF, interferons (α, β, or γ) and IL-

2) to provide a sufficient quantity of cells for therapeutic use (*see* Sekine at page 4, lines 4-18, page 4, line 47 to page 5, line 16, and claims 1, 4, 8, and 9).

Further, Sekine notes that it was unexpectedly found that both the second and third step (*i.e.,* the culturing of the CD3-activated peripheral blood lymphocytes in an uncoated culturing substrate like an uncoated culture flask and expanding the culture using a lymphokine like IL-2 in (high) concentrations of about 200 U/ml to about 2000 U/ml) provides a superior increase in cell proliferation number (*see* Sekine at page 3, lines 6-7, page 3, lines 35-37, page 3, lines 40-41, page 4, lines 51-53, and page 5, lines 17-18). The cell number of the starting cell population may be increased by a factor of more than 1000 up to a factor of about 10,000 to 30,000 (*see* Sekine at page 3, lines 15-18, claim 2, and page 6, lines 14-19). The resulting proliferated cell population consists almost only of T cells (due to the T cells capability of fast proliferation) (*see* Sekine at page 6, lines 16-18), and the proliferation is achieved completely independent of monocytes (*see* Sekine at page 3, line 34).

In contrast, the presently claimed method provides cascade primed cells (CAPRI cells) by stimulating PBMC with immobilized anti-CD3 antibodies to produce primary stimulated PBMC, and then by adding naïve PBMC to the primary stimulated PBMC, and finally incubating the naïve PBMC in the presence of the primary stimulated PBMC to stimulate the naïve PBMC by the primary stimulated PBMC.

Thus, the claimed method differs from the primary reference, Sekine. at least in that naïve PBMC are (i) added to the CD3 primary stimulated PBMC and (ii) the naïve PBMC are incubated in the presence of the CD3 primary stimulated PBMC. These distinguishing features enable the production of CAPRI cells which are capable of efficiently lysing cancer cells, such as breast cancer cells, in a MHC-restricted manner (*see* page 3, lines 13-15, page 3, line 23 to page 5, line 22, and Figures 1 and 2 of the manuscript of Dr. Wank included with

the 37 C.F.R. § 1.132 declaration filed with the response of June 17, 2009 (hereinafter "Declaration"); *see also* page 15, line 29 to page 16, line 23 of the application as-filed).

In contrast to the cited references, the present invention provides a method of treating cancer which comprises providing stimulated PBMC that are able to efficiently lyse cancer cells in a MHC-restricted manner, by way of a method as defined in claim 29, comprising steps (a) to (c) to provide cascade primed cells (CAPRI cells) and administering the CAPRI cells into a cancer patient in step (d).

In contrast, Sekine seeks to address the technical problem of efficiently proliferating peripheral blood lymphocytes, in particular T cells. The solution provided by Sekine is a non-specific activation and proliferation of T cells using immobilized anti-CD3 antibodies. In accordance with a non-specific activation, the proliferation is completely independent of monocytes (*see* Sekine at page 3, line 34). Thus, the cell population obtained by the method described in Sekine is not primed and imprinted by tumour-specific peptides but is generated utilizing a non-specific activator, *i.e.* an anti-CD3 antibody.

To this end, Sekine discloses that the obtained proliferated cell population exhibits an increased cytotoxicity for the leukaemia cell line K562. This particular cell line is known to show no or only minimal expression of MHC (HLA) antigens and are therefore killed by NK cells which specifically target for killing those cells that have no or diminished MHC expression. However, as most cancer cells express MHC molecules, NK cells will not target and destroy most cancer cells (*see* page 5, lines 19-22 of Dr. Wank's manuscript included with the Declaration, and page 363 of the immunology textbook "Kuby Immunology" (6th ed., 2006, W.H. Freeman and Company) which was included with the Declaration, as well as Kronenberg, M., Toward an understanding of NKT cell biology: progress and paradoxes, *Ann. Rev. Immunol.* 23:877-900 (2005)).

Thus, the methods of Sekine are not the same and do not achieve the effects of the presently claimed methods, *i.e.* the provision of cells capable of efficiently lysing cancer cells in a MHC-restricted manner. Rather, Sekine merely refers to the proliferation of peripheral blood lymphocytes. Accordingly, the skilled artisan would not have looked to Sekine for motivation when seeking means to provide cells that efficiently destroy cancer cells in an MHC-restricted manner.

Even if one of skill in the art would have looked to Sekine, there is no suggestion whatsoever in Sekine regarding the steps of adding naïve PBMC to CD3 primary stimulated PBMC and incubating the naïve PBMC in the presence of the CD3 primary stimulated PBMC. Rather, Sekine specifically teaches to stimulate and proliferate PBMC in the presence of immobilized anti-CD3 antibodies, followed by transferring the obtained proliferated PBMC to a reaction substrate without anti-CD3 antibodies for further cultivation, and finally adding a lymphokine to further increase the cell number and obtain a proliferated cell population consisting mainly of T cells (*see* the passages of Sekine cited above). Thus, as already stated, Sekine only relates to a method for proliferating PBMC, in particular T cells, using anti-CD3 antibodies and lymphokines for the purpose of obtaining a sufficient quantity of cells for use in adoptive immunotherapy, but neither teaches nor suggests using CD3 stimulated PBMC to stimulate and imprint naïve PBMC for the purpose of obtaining PBMC capable of efficiently lyse cancer cells in a MHC-restricted manner.

The Office has cited Flyer in combination with Sekine against the present claims. However, Applicants note that Flyer fails to cure the deficiencies in Sekine described above. No disclosure can be found in Flyer regarding the cascade priming method as defined in steps (a) to (c) of claim 29. Rather, Flyer merely teaches that the non-specific activation of T cells via anti-CD3 antibodies results in the secretion of various cytokines like IL-2 that promote T

cell proliferation (see page 40, line 32 to page 41, line 29). In this regard, the Office states "Thus, given the reference teachings it would have been obvious to one of ordinary skill in the art that either the third, or the second and third steps of Sekine could be favorably supplanted by a single step comprising incubating the anti-CD3 stimulated patient derived PBMC with naïve PBMC in the presence of immobilized anti-CD3 as taught by Flyer". However, naïve PBMC (non-stimulated PBMC; *see* page 1, line 10 of the application as-filed) do not secrete cytokines, and the second step and third step of Sekine do not include anti-CD3 antibodies. Thus, there is no reason at all for the skilled person to add naïve PBMC to the second and/or third step of Sekine, contrary to the opinion of the Office.

Further, the disclosure of Flyer would not have prompted the skilled artisan to add naïve PBMC in one of the steps of the method described in Sekine, because the method of Sekine is directed to the production of a large quantity of proliferated cells from a small starting population of peripheral blood lymphocytes. Thus, the addition of naïve PBMC actually runs contrary to the objective and disclosure of Sekine.

Furthermore and importantly, the skilled artisan, taking into account the teaching of Flyer, would not have added naïve PBMC to one of the steps of the Sekine method because it would be expected that the inclusion of naïve PBMC to one of the steps of the Sekine method would merely result in a proliferation of the added naïve PBMC, because the method of Sekine is directed to expanding or proliferating a small starting cell population to obtain a large quantity of proliferated cells. In other words, one of skill in the art would have expected that the addition of naïve PBMC would not change the nature of the Sekine method of proliferating PBMC, except that the proliferation method involves a higher starting number of PBMC. Consequently, the skilled artisan, faced with the problem of providing cells that are able to efficiently lyse cancer cells in a MHC-restricted manner, would not have been

prompted by the mere teaching of Flyer that non-specifically CD3-stimulated T cells secrete cytokines to add naïve PBMC to one of the steps of the method of Sekine in reasonable expectation of generating cells that can lyse cancer cells in a MHC-restricted manner. The skilled artisan would instead have expected that the addition of naïve PBMC would not be able to provide cells that are able to efficiently lyse cancer cells in a MHC-restricted manner and, thus, would not have contemplated to add naïve PBMC to one of the steps of the Sekine method.

In summary, one of skill in the art, taking into account the combined teachings of both Sekine and Flyer, would not have been able to arrive at the claimed method because (a) the skilled artisan would not have looked to Sekine at all for the reasons set out above, (b) neither Sekine nor Flyer teaches or suggests to first produce primary stimulate PBMC by stimulating PBMC with anti-CD3 antibodies and then to use the primary stimulated PBMC for incubation with added naïve PBMC resulting in a stimulation of the added naïve PBMC by the primary stimulated PBMC, and (c) the teaching of Flyer that CD3 stimulated T cells secrete cytokines would not have incited the skilled person to add naïve PBMC to PBMC stimulated by anti-CD3 antibodies for the reasons set out in detail above.

Applicants again emphasize that the present invention provides the unexpected finding that the CAPRI cells of the present invention are far more effective in the destruction of tumor cells than are CD3-activated PBMC. This cytotoxicity is MHC restricted (*see* page 15, line 29 to page 16, line 23 of the application as filed, and Figures 1 and 2 of Dr. Wank's manuscript included with the Declaration). It was also surprisingly found that CAPRI cells lead to increased MHC class I and MHC class II expression by tumour cells, thereby allowing T effector cells to more efficiently recognize the tumour cells (*see* Figure 3 of Dr. Wank's manuscript included with the Declaration). These advantageous effects associated with the

CAPRI cells of the present invention would not have been expected or foreseen by the skilled person before the priority date of the present application. These effects were entirely unexpected and therefore further support the non-obviousness of the claimed method.

By way of further support attached is a revised version of the previously provided manuscript by Dr. Wank (now entitled "T cell-activated monocytes prime naïve T cells against autologous cancer cells: adoptive transfer prolongues patients life") (herein, Exhibit A). The revised manuscript provides survival data of breast cancer patients (*see* pages 11 and 23-24 of the revised manuscript), as well as *in vivo* data demonstrating the CAPRI cells' ability to destroy breast cancer and colorectal cancer cells in a mouse model. Also attached as Exhibit B is in vivo data showing the ability of the CAPRI cells of the present invention to destroy breast and colorectal cancer cells in a nude mouse model.

In the last paragraph of section 8 of the Office Action, the Office argues that the evidence of unexpected results is not found convincing because Applicant does not appear to have compared the claimed method to the closest prior art which was Babbitt or Gold. In response, Applicants attach a table prepared by Dr. Wank (herein, Exhibit C). This table summarizing the differences between the CAPRI method and the prior art methods according to Babitt *et al.* and Gold *et al.* (entitled "Adoptive cell therapy of autologous cancer with autologous immune cells from the peripheral blood (PBMC)". The CD3 activated PBMC according to Dr. Wank's manuscript included with the Declaration are non-specifically activated and stimulated by the monoclonal anti-CD3 antibody OKT3 and, as a result, secrete various cytokines. In the Babbitt reference, the supernatant of PBMC that have been non-specifically activated and stimulated with the same anti-CD3 antibody (in Babbitt this supernatant is referred to as "T3CS") is added to naïve PBMC. The result of both protocols, however, is the same in that PBMC are proliferated utilizing cytokines secreted by CD3-

activated PBMC. Thus, the experimental results described in Dr. Wank's manuscript are considered to represent a fair comparison between CAPRI cells and "conventional CD3 activated cells". Moreover, the method of Babbitt appears similar to the CD3-activation of Dr. Wank's manuscript because Babbitt's supernatants are monitored for sufficient amounts of anti-CD3 antibodies and supplemented with fresh anti-CD3 antibodies as needed. However, in view of the observed drastically different cytotoxic effect of CAPRI cells of the present invention and CD3-activated cells as known in the prior art on autologous cancer cells (nearly complete lysis vs. no lysis; *see* Figure 1 of Dr. Wank's manuscript included with the Declaration), the superior cytotoxic activity of the CAPRI cells of the present invention is evident.

On page 7 of the Office Action, the Office states that Applicants has not provided evidence that that method of Babbitt results in internalization of CD3 and αß TCR. In support, Applications provide Alcover *et al.* (*Critical Reviews in Immunology*, 20:325-346 (2000)) and Liu *et al.* (*Immunity*, 13:665-675 (2000)). These references also support Applicants' argument that anti-CD3 antibodies suppress T cell activation by way of the αß TCR because of the internalization of both CD3 and the αß TCR.

Claim 33 stands rejected under 35 U.S.C §103(a) as purportedly unpatentable over Sekine in view of Flyer, and further in view of Granger (U.S. Patent No. 5,837,233) and Johnson (U.S. Patent No. 5,217,704). As claim 33 depends from claim 29, Applicants submit that the method as defined in claim 33 is patentable over the cited references for the same reasons presently above in connection with claim 29. This this end, Granger and Johnson do not remedy the deficiencies of Sekine combined with Flyer. The Office notes that Sekine and Flyer do not disclose teaching the administration of activated PMBCs into a tumor, and thus cites Granger and Johnson in this regard. However, neither Johnson nor Granger disclose or

suggest to first produce primary stimulated PBMC by stimulating PBMC with anti-CD3 antibodies and then use the primary stimulated PBMC for incubation with added naïve PBMC. Thus, the cited references in combination do not disclose the presently claimed invention.

Accordingly, Applicants request that these rejections be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully submit that the application is in condition for allowance. Applicant respectfully request favorable consideration and prompt allowance of the application.

If there are any questions regarding this response or the application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response, and please charge any deficiency in fees or credit any overpayments to Deposit Account No. 05-1323 (Docket # 104341.B090019).

Respectfully submitted,

December 7, 2009

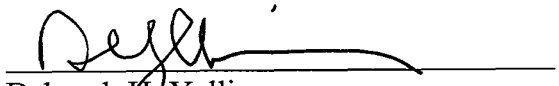

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EXHIBIT A

T CELL-ACTIVATED MONOCYTES PRIME NAÏVE T CELLS AGAINST AUTOLOGOUS CANCER CELLS: ADOPTIVE TRANSFER PROLONGUES PATIENTS LIFE

Until now adoptive cell therapy often used immunogenic material of tumors to induce cancer-specific cytotoxic lymphocytes. We developed a novel priming method without tumor cells or identified peptides presuming that, first, monocytes and dendritic cells of cancer patients harbour tumor-immunogenic information; second, that these monocytes/dendritic cells, if stimulated, prime naïve/resting T cells to tumor-specific effector cells; third, that activated immune cells can intensify pMHC expression of cancer cells. Depletion experiments revealed $CD14^+$ monocytes as main collectors of tumor-immunogenic information. $CD14^+$ monocytes, stimulated with CD3-activated T cells and cocultured with naïve T cells expressing the $\alpha\beta$-TCR, matured rapidly to $CD1a^+$ $CD83^+$ dendritic cells and primed $CD3^+CD4^+$ and $CD3^+CD8^+$ to cytotoxic effector cells. This CAscade PRimed (CAPRI) immune cell quartett showed a significant numerical decrease of suppressive $CD25^{high}$ $FOXp3^+$ regulatory T cells and induced a marked upregulation of MHC class I and class II expression in cancer cells, crucial for autoimmune-like lysis by autologous $CD8^+$ and $CD4^+$ T cells. We show clinical evidence of the CAPRI cell concept in SCID mice and in an adjuvant intention-to-treat setting of breast cancer patients with metastasis (N=42, no CAPRI treatment N=428). Almost double the number of patients than expected survived five years (p=0.0001)

Introduction

Numerous therapeutical modalities have been developed to hinder growth or to induce destruction of malignant tumor cells. The multitude of modalities reflects the inexhaustible number of strategies of cancer cells to evade control by immune cells. But the immune system seems to be able to develop counter strategies. For example, not all women infected with cancer-associated human papilloma viruses (HPV) develop carcinomas. Women with a certain immunogenetic endowment, i.e., with certain HLA alleles, are at high risk, whereas women with other HLA alleles are at low risk to develop this cancer[1,2]. HLA alleles and the encounter with a certain HPV type were decisive for development of cervical cancer[3,4]. Unrecognized immune responses must prevent the rise of carcinoma cells in women carrying resistance-associated immune response genes of the HLA system[5]. Immune surveillance of cancer growth by T lymphocytes necessarily includes recognition of tumor-immunogenic peptides. To induce such T cells, recent strategies have focussed on the use of dendritic cells that have been incubated in vitro with tumor cell lysates, pulsed with defined tumor peptides or transfected with RNA or DNA from tumor cells[6,7].

Gene mutations and corresponding mutated cellular proteins can be recognized as "tumor markers" in early stages of cancer development. For example, mutations of the p53 gene have been identified as free circulating DNA in pre-cancer and cancer patients[8,9]. Cytotoxic T cell responses to different and differently mutated tumor targets have been reported[10-16]. We have been interested to identify conditions, which would stimulate antigen presenting cells (APC) to process, express and transfer tumor antigen information to naïve T cells, leading to their differentiation into T effector cells and preventing their inactivation or deletion by cancer cells, as observed in tumor infiltrating lymphocytes[17,18].

We decided to activate APC by activating T cells in PBMC cultures with the monoclonal antibody OKT3 for the following reasons: activated T cells produce a wealth of cytokines such as the monocyte-activating interferon γ (IFNγ), which stimulate intracellular processing as well as strong expression of the pMHC and of costimulatory molecules. Activated APC, however, may not be able to deliver the tumor-immunogenic message to CD3-activated T cells, since ligation of CD3 chains by OKT3 antibodies induces internalization of the CD3/TCR complex[19,20]. We therefore added to the CD3-activated cells unstimulated autologous PBMC as a source of naïve T cells which could respond to the tumor-specific information from the activated APC. This approach induced a cascade of events leading to the priming of naïve T cells. We coined the procedure cascade priming (CAPRI) and the actively involved immune cells CAPRI cells.

Here we show that CAPRI cells are much more effective in the destruction of malignant tumor cells then are CD3-activated cells and this cytotoxicity is MHC restricted. Cascade priming induced rapid differentiation of monocytes to dendritic cells and enhanced expression of costimulatory molecules. In addition, CAPRI cells lead to increased MHC class I and MHC class II expression by malignant tumor cells, thus allowing both CD4 and CD8 T effector cells to recognize and to lyse cancer cells. We show that the optimal cancer cell lysis observed with CAPRI cells results from the cooperation of a cellular quartett consisting of T helper cells, T cytotoxic cells, dendritic cells and monocytes. In depletion experiments we will show that monocytes were absolutely required in the priming phase, but not during cancer cell destruction, whereas dendritic cells were not absolutely needed in both phases but contributed significantly to priming and to cancer cell destruction. We will show that $CD4^+$ and $CD8^+$ T cells are absolutely required for priming and cancer cell destruction, and that the presence of $CD4^+$ T cells in the phase of cancer cell

destruction cannot be replaced by CAPRI culture supernatants indicating that $CD4^+$ helper T cells act in this phase as cytotoxic effector cells.

Finally, we will show efficient lysis of cancer cells transplanted into SCID mice, destruction of two melanoma lesions in a patient by locally injected CAPRI cells and the significantly increased five-year survival in an adjuvant intention-to-treat setting of forty-two breast cancer patients with metastasis treated with CAPRI cells, compared to CARI cell untreated patients of the Munich Tumor Center.

RESULTS

Nearly complete lysis of autologous cancer cells within 24 h by CAPRI cells but not by CD3-activated cells.

We stimulated APC in PBMC bulk cultures by activating T cells with the OKT3-CD3 antibody, which binds to a conformational epitope expressed either by CD3-$\varepsilon\delta$ or CD3-$\varepsilon\gamma$ chains[22]. Activated T cells produce monocyte activating cytokines such as IFNγ which can lead to activation of APC. Since ligation of CD3 molecules downmodulates the CD3:$\alpha\beta$TCR complex by internalization or by prevention of recycling[19,20] (not shown), we added autologous unstimulated PBMC to CD3-activated PBMC to provide a source of naïve T cells expressing the antigen TCR. After one day in culture CAPRI cells were compared with CD3-activated cells for their ability to kill autologous cancer cells.

The destruction of cancer cells by CAPRI and by CD3-activated cells was measured by microscopic inspection (**Fig. 1a,b,c,d,e,f**) and by the chromium[51] release assay (**Fig. 1g**). Figure 1 shows an example of lysis of breast carcinoma cells. Microscopic inspection of the tumor cells indicated no or little reduction of cancer cell numbers after 20h coculture with CD3-activated cells (**Fig. 1e**) compared with carcinoma

cultures at time 0 (**Fig. 1a,b**), whereas lysis of most cancer cells was observed after 20h of coculture with CAPRI cells (**Fig. 1f**). In chromium^{51} release assays CD3-activated PBMC showed no significant lytic activity (**Fig. 1g**), while CAPRI cells lysed 27.1% of cancer cells at a 5 :1 effector to target (E:T) ratio and 89.9% of cancer cells at a E:T ratio of 20:1 (**Fig. 1g**). Furthermore, CAPRI cells could be successfully reseeded onto new autologous cancer cell lawns without addition of cytokines (not shown). Similar results were observed in many patients with different malignant tumor types (**Table 1**)

HLA class I and HLA class II antibodies abrogated lysis of cancer cells and lysis of allogeneic cancer cells required HLA sharing.

Generation of cytotoxic T cells depends on interactions between the αβ TCR and the pMHC[24]. MHC-restricted interactions were analysed using allogeneic cells and antibodies blocking the pMHC complex. Figure 3 shows results of CAPRI cells from two unrelated breast cancer patients with defined HLA class II DQ alleles. These CAPRI cells were tested with breast cancer cells from 6 unrelated female patients (**Fig. 2a**). The degree of lysis was estimated microscopically after 24h of coculture using the HLA microcytotoxicity scale (see materials and methods). CAPRI cells lysed autologous cancer cells strongly and allogeneic cancer cells with shared class II HLA-DQ alleles approximately half as well, whereas lack of HLA-DQ sharing resulted in only minimal background lysis (**Fig. 2a**). This suggested that HLA class II surface molecules of APC presented tumor-immunogenic peptides, but complete lysis may depend on sharing of both HLA class I and HLA class II antigens. This was indirectly supported by blocking lysis of autologous cancer cells with antibodies against HLA class I and class II molecules. Lysis of cancer cells by CAPRI cells (**Fig. 2c**) was strongly diminished either with the antibody W6/32 binding to all HLA class I

molecules (**Fig. 2e**) or with the antibody CIA2 binding to the HLA-DQ class II molecules (**Fig. 2d**). The required concurrence of HLA class I and class II presentation indicated a comprehensive interdependence of helper and cytotoxic T cells for successful lysis of cancer cells. As expected, CAPRI cells showed only very weak activity against the NK target cell K562, which usually does not express HLA antigens (not shown). This very weak lytic activity may come from activated NKT cells in the PBMC cultures[25].

CAPRI cells enhanced HLA class I and HLA class II surface expression in epithelial and other solid malignant tumor cells

CAPRI cells lysed cancer cells in an HLA restricted manner (**Fig. 2**). Given that cancer cells tend to downregulate HLA expression to evade recognition by cytotoxic T cells[26,27], we questioned whether CAPRI cells upregulate HLA expression by cancer cells. We compared the HLA expression of CFSE (5(6)-carboxy fluorescein diacetate N-succinimidyl ester) labelled cancer cells after coculture with autologous unstimulated PBMC, with CD3-activated PBMC or with CAPRI cells using a low effector to target ratio of 5:1 (**Fig. 3**). Increased mean fluorescence intensity (MFI) of gated living cancer cells could be observed only in cocultures with CAPRI cells, but not in cocultures with CD3-activated PBMC or with unstimulated PBMC (**Fig. 3**). CAPRI cell stimulated cancer cells showed a 40% increase in HLA class I expression (MFI vs MFI) and a 60% increase in HLA-DR class II expression (MFI vs MFI) (**Fig. 3a**). Enhanced MHC class II expression of cancer cells could be a deciding factor in the destructive power of CAPRI cells, since interactions between MHC class II and CD4 molecules are pivotal for the augmentation of cytotoxic T cell responses[28,29].

Maturation of monocytes to dendritic cells during the CAPRI procedure

Stimulated APC express high levels of B7 and other costimulatory molecules as well as MHC class I and MHC class II molecules[21]. We assumed that APC in PBMC bulk cultures become stimulated by CD3 activation of T cells and investigated whether differences could be observed between APC in CD3-activated PBMC and CAPRI cells. We compared phenotypic changes of monocytes during CD3-activation of PBMC with monocytes during the cascade priming procedure (**Fig. 4b**) by adding CFSE-labelled purified $CD14^+$ cells as tracer to the PBMC at day 0. Phenotypic markers of CFSE-labelled $CD14^+$ monocytes were determined before the start of activation (day 0), after one day (day 1) and after five days (day 5) (**Fig. 4**). In CAPRI cells a considerable number of monocytes lost CD14 expression and matured as defined by the acquisition of the dendritic cell markers CD1a and CD83 at day 1 and stronger at day 5 (**Fig. 4b**). Upregulation of the costimulatory molecules CD80, CD86 and CD40 as well as HLA-DR class II and HLA class I molecules was also observed (**Fig. 4b**). In CD3-activated PBMC the number of $CD14^+$ monocytes remained constant, no numerical increase of cells expressing CD83 and CD1occured. Upregulation of costimulatory molecules CD80, CD86, CD40 as well as of HLA class I and of HLA-DR was observed but was clearly lower than in CAPRI cell cultures (**Fig. 4c, Supplementary Table 1** online). Quantitative analysis of the leucocyte subpopulations in CD3-activated PBMC and CAPRI cells from five cancer patients confirmed these observations. We found significantly increased numbers of matured dendritic cells in CAPRI cells as compared to CD3-activated PBMC (paired t-test, P=0.000096, **Supplementary Table 1** online) and a higher percentage of monocytes in CD3-activated PBMC as compared to CAPRI cells on day 5 (paired t-test, P=0.023, **Supplementary Table 1** online).

Depletion of monocytes, dendritic cells, T helper or T cytotoxic cells from PBMC or CAPRI cells reduced or forestalled T cell priming or cancer cell lysis

Depletion of $CD3^+CD8^+$ T lymphocytes, $CD3^+CD4^+$ T lymphocytes, $CD14^+$ monocytes or $CD83^+$ dendritic cells, but not of $CD19^+$ B lymphocytes (not shown) affected T cell priming or lysis of cancer cells. The influence of depletion was analysed at the following time points: 1) from unstimulated PBMC before CD3-activation, 2) from unstimulated PBMC, to be added to CD3-activated PBMC, 3) from CAPRI cells before coculture with cancer cells (**Fig. 5**).

Not unexpectedly, depletion of $CD3^+CD8^+$ T lymphocytes at each time point prevented development of any lytic capacity of CAPRI cells (**Fig. 5d**, PBMC, P_{slope} = 0.003, $P_{intercept}$ = 0.001; reimported PBMC, P_{slope} = 0.003, $P_{intercept}$ = 0.001, CAPRI effector cells P_{slope} = 0.003, $P_{intercept}$ = 0.003). Depletion of $CD4^+$ T cells at each time point had the same effect. Thus $CD3^+CD4^+$ T cells were required during the priming phase and also absolutely required in the effector phase (**Fig. 5c**, PBMC, P_{slope} = 1.25×10^{-7}, $P_{intercept}$ = 0.022; reimported PBMC P_{slope} = 1.25×10^{-7}, $P_{intercept}$ = 0.022, CAPRI effector cells P_{slope} = 4.6×10^{-7}, $P_{intercept}$ = 0.018. Depletion of $CD14^+$ monocytes prior to anti CD3-activation or from PBMC added to CD3-activated cells as source of naïve T cells, completely prevented the development of lytic activity of CAPRI cells, P_{slope} = 0.003, $P_{intercept}$ = 0.001 and P_{slope} = 0.003, $P_{intercept}$ = 0.001 (**Fig. 5a**). This absolute requirement of monocytes as source of tumor information sheds new light on the enigma of failing immune responses against cancer cells. After priming naïve of T cells to effector cells, during cancer cell destruction, $CD14^+$ cells did not support significantly lysis of cancer cells (P_{slope} = 0.37, $P_{intercept}$ = 0.057). Depletion of $CD83^+$ dendritic cells at each time point reduced the development of CAPRI lytic activity by half (PBMC, P_{slope} = 0.00072, $P_{intercept}$ = 0.008), reimported PBMC, P_{slope} = 0.004, $P_{intercept}$ = 0.007, CAPRI effector cells P_{slope} = 0.01, $P_{intercept}$ =

0.004) (**Fig. 5b**). The "only medium" importance of dendritic cells for priming suggests that fewer of them collected tumor-immunogenic information. However, during cancer cell destruction a significant "medium" contribution of dendritic cells to the lytic capacity of CAPRI cells may indicate a continuous supply of contact-information and/or of cytokines to T effector cells during cancer cell destruction. Maintaining a flow of information or a threshold of activating signals may prevent inactivation of effector cells, as observed in TIL. Cancer cells often express only low levels of MHC molecules or activation molecules. Failing immune responses as a consequence of rudimentary immunogenic information by cancer cells have been demonstrated[26,27].

$CD4^+$ T lymphocytes cannot be replaced by supernatants from CAPRI cell cultures.

CAPRI culture supernatants were added to CAPRI cell cultures to clarify, whether $CD4^+$ T lymphocytes provide only "cytokine help" to cytotoxic $CD8^+$ T cells or participate as effector cells in the cancer cell destruction. To avoid depletion of $CD14^+CD4^+$ monocytes, $CD3^+$ cells were first isolated from PBMC (1), then $CD4^+$ cells were depleted. The $CD4^+$ depleted CD3-isolate was added to (1). Supernatants were added before CD3-activation or from unstimulated PBMC, which were added in the second step to supply naïve T cells expressing the antigenTCR. In both steps of cascade priming supernatants could not replace the presence of $CD4^+$ T cells. Cancer cells were only minimally destroyed in the absence of $CD4^+$ T cells indicating their cytotoxic activity during cancer cell lysis (Suppl Fig. on line)

The numbers of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes decreased during cascade priming

Several reports described regulation of immune cell homeostasis and suppression of cytolytic immune responses against human cancer cells by $CD4^+CD25^+$ regulatory T cells[30-36]. Modulation and suppression appeared antigen-specific or non antigen-specific and restricted to $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes[30-36]. We compared the percentage of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes of CD3-activated cells and of CAPRI cells. The number of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes declined only in CAPRI cell cultures (**Supplementary Fig. 1** online). This could indicate a suppressive role of some $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes. CD3-stimulation of T lymphocytes activates different pathways leading to a stronger Foxp3 expression than stimulation of the antigen TCR[37], which is addressed in the cascade priming procedure. Dendritic cells can abrogate the regulatory activity of $CD4^+CD^{25+}$ T lymphocytes of the human peripheral blood[38]. This effect could be enhanced by activated dendritic cells. Experiments will have to clarify whether a subpopulation of $CD4^+CD25^{high}/Foxp3^+$ T lymphocytes like IL-10 producing cells[36] or different $Foxp3^+$ isoforms were eliminated or neutralized during the cascade priming procedure.

Successful lysis of two melanoma lesions by infiltration of CAPRI cells at the tumor border

CAPRI cells were injected at the border of the melanoma in order to attack the growth zone of melanoma. Fig. shows that within three months both lesions disappeared. However, the melanoma patient had already systemic metastatic disease as the CAPRI cell therapy was initiated. The limited number of CAPRI cells generated against a huge tumor mass could not prevent that she finally died from her tumor.

Comparison of CAPRI cell treated breast cancer patients with breast cancer patients ot the Munich Tumor center, not treated with CAPRI cells

Patients of the Munich Tumor Center and patients treated additionally with CAPRI cells in ajuvant fashion were analysed using the Kaplan Meyer statistics. CAPRI cell treated patients were included in the analysis irrespective of other therapeutic modalitis, if they had the same tumor status, (i.e., distant metastasis, Fig.) at the begin of CAPRI cell treatment. For inclusion in the analysis patients had to receive at least 500 x 10^6 CAPRI cells. Most patients received many more cells ( ). Ideally, patients should receive 50-80 x 10^6 CAPRI cells three times per week for one year. Dosis and frequency of injections was then reduced, if PET/CT analyses showed destruction or loss of activity of former cancer locations. Despite a considerable variation in frequency of injections and of cell numbers, not desired but not avoidable in intention-to-treat settings, a significant increase of survival emerged for CAPRI cell treated patients (, Fig.). There were no adverse reactions from the CAPRI cell therapy observed, on the contrary, patients reported that they overcame adverse reactions after chemotherapy much better. Most times paients could resume professional activities already one day after the chemotherapy.

Discussion

The dramatic power of autologous MHC-restricted immune responses, first recognized by Zinkernagel and Doherty[39], contrasts with the failure of immune surveillance of MHC-restricted tumor infiltrating lymphocytes (TIL) in cancer patients. However, it seems that TIL can be revived in vitro. A recent review shows clearly that adoptive cell therapy (ACT) with TIL can be successfully applied in patients[40].

Adoptive immunotherapy using atologous TIL and IL-2 in combination with non-myeloablative chemotherapy and total body irradiation achieved in seven out of 25 patients (28%) complete responses[40]. This represents certainly an important progress in adoptive immunotherapy, but calls for further improvements.

One factor of the failing immune response against cancer cells appears particularly important. It is the unprofessional presentation of tumor-immunogenic peptides by cancer cells to naïve T cells as well as their lack of HLA class II expression and of costimulatory molecules, which entails often inactivation of naïve T cells. To instruct T cells before the encounter with cancer cells, dendritic cells have been loaded with tumor antigens or transfected with RNA encoding tumor antigens. We believed that APC in the peripheral blood of cancer patients do not necessarily need further peptide loading or RNA transfection to prime naïve T cells against autologous cancer cells because they harbour sufficient tumor-immunogenic information.

In order to optimize tumor-immunogenic information to be transfered from APC to T cells we tried to enhance the expression of HLA and costimulatory molecules as follows: we first stimulated T cells in PBMC bulk cultures with the OKT3 anti-human antibody. This stimulation of APC via CD3-activated T cells resulted in the desired enhanced expression of MHC and costimulatory molecules (**Fig. 4**). But this required another manipulation, since the message of these APC could not be further passed to, and received by, the CD3-activated T cells. As previously reported, CD3-activation downmodulates the TCR complex by internalization or by prevention of recycling[19,20]. To overcome this obstacle we added resting/naïve T cells, which are present in PBMC bulk cultures and express high levels of the TCR. The engagement of stimulated APC with unstimulated/naive T cells expressing the αβTCR yielded the expected effect in generating helper and cytotoxic T effector cells that lysed efficiently autologous cancer cells in vitro (FIG). This profound superiority of CAscade PRImed

(CAPRI) cells compared to CD3-activated PBMC was demonstrated in the lytic performance against autologous cancer cells. While CAPRI cells lysed autologous cancer cells very efficiently after 18-24 hours, the CD3-activated PBMC did so only to a minimal non-significant cancer extent (**Fig...**) These results confirmed our notion that APC of cancer patients in principle harbour sufficient tumor-immunogenic information, and, if primed alike in the protocol we designed and used herein, such APC could differentiate unstimulated/naïve T cells to T effector cells.

Although these studies suggested strongly αβTCR-pMHC interactions, the role of HLA class I versus HLA class II presentation of tumor-immunogenic determinants needed more clarification. Three sets of experimental results obtained in our present study support the notion that the tumor-immunogenic information was most likely communicated by the pMHC. First, allogeneic cancer cells were lysed by CAPRI cells only if CAPRI cells and allogeneic cells shared HLA molecules, as shown for HLA-DQB*0201 (**Fig. 2a**). Second, anti HLA class I as well as anti HLA class II antibodies strongly reduced lysis of breast cancer cells by CAPRI cells (**Fig. 2b-d**). Interestingly, the HLA class I and class II antibodies blocked lysis equally efficient, suggesting the participation of both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells in the MHC-restricted cancer lysis by CAPRI cells. Third, lysis by CAPRI cells of the NK target cells K562, a chronic myeloid leukemia cell line with no or lowgrade MHC expression, was minimal (not shown).

Many signalling pathways are initiated by engagement of the antigen-TCR by the pMHC, but full T cell activation requires costimulatory signals. We therefore compared the expression of costimulatory molecules and of MHC molecules in APC of CAPRI cells and in APC of CD3-activated PBMC by following a possible maturation of CFSE-labelled $CD14^+$ monocytes in both cultures (**Fig. 4**). The numbers of CFSE-labelled $CD14^+$ monocytes decreased in CAPRI cell cultures,

whereas the numbers of CFSE-labelled cells with the $CD1a^+CD83^+$ mature dendritic cell phenotype increased (**Fig. 4b**). In contrast, the numbers of $CD14^+$ monocytes did not decrease in CD3-activated PBMC (like in unstimulated PBMC), and the numbers of $CD1a^+CD83^+$ dendritic cells did not increase (**Fig. 4a,c**). Paired t-tests, comparing numbers of monocytes and dendritic cells of five patients, confirmed the significant difference between CD3-activated PBMC and CAPRI cells (P=0.000096, **Supplementary Table 1** online). Furthermore, CFSE-stained cells within CAPRI cell cultures showed an increased expression of CD40, CD80 and CD86 and HLA molecules which support priming of naïve T cells after one and more pronounced after five days (**Fig. 4**).

Summing up, only in CAPRI cells cultures monocytes and dendritic cells significantly increased expression of costimulatory and MHC molecules and dendritic cells increased numerically. Both effects contributed to a differentiation of naïve T cells to potent tumor-specific $CD4^+$ and $CD8^+$ effector T cells that could efficiently lyse autologous cancer cells.

The CAPRI procedure enhanced tumor-immunogenic information of APC by enhancing MHC expression, but carcinomas often escape recognition by downregulation of HLA expression[26,27]. We therefore examined HLA expression of CFSE-stained carcinoma cells cocultured with either CAPRI cells, or CD3-activated PBMC or unstimulated PBMC. Clearly, only CAPRI cells were able to increase expression of HLA class I and class II in autologous cancer cells, either by cell contact or cytokines or both (**Fig. 3**). Upregulation of HLA class I and class II expression in carcinoma cells appeared to be another key determinant of the destructive power of CAPRI cells and raised the question of the true contribution of the different immune cell subpopulations in different phases of the priming process

and the lytic phase. We therefore evaluated the contribution of different immune cell populations during the cascade priming process and during the subsequent lysis of cancer cells. We did so by depleting systematically different cell types from PBMC either before their CD3-activation, or from unstimulated PBMC before their addition to already activated PBMC, or from CAPRI cells before cancer cell lysis (**Fig.5**). As expected, no lysis of cancer cells occured after $CD8^+$ T cell depletion at any time point (**Fig.5**). Interestingly, this requirement for presence during all stages of priming as well as during cancer cell lysis, was also true for $CD4^+$ T helper cells (**Fig.5**). So only if $CD4^+$ T cells and $CD8^+$ T cells were present, the highly efficient lysis of cancer cells was achieved (**Fig.5**). Presence of $CD4^+$ T cells could not be replaced with supernatants from undepleted CAPRI cells added to $CD4^+$ T-depleted CAPRI cells, indicating a significant cytotoxic activity of $CD4^+$ T cells against cancer cells (**Fig**)

Depletion of APC populations revealed no contribution of B lymphocytes to the destructive capacity of CAPRI cells at any time point (not shown), but of monocytes and dendritic cells. Most importantly, $CD14^+$ monocytes and not dendritic cells were absolutely required during the cascade priming procedure, from PBMC before CD3-activation in the first step, and in the second step from unstimulated PBMC before coculture with CD3-stimulated PBMC. Although speculative at this point, it looks like that capture of tumor material silences monocytes *in vivo* and prevents their differentiation to optimal presenting cells, the dendritic cells. So far failing immune responses have been explained mainly by inactivation of T cells at the tumor site but not by mute monocytes. Mute monocytes could be the bottle neck in the initiation of immune responses against cancer cells *in vivo*. At the moment we do not know whether the activated monocytes per se, or the activated monocytes *in transition* of differentiation, or rather *de novo* matured dendritic cells are the crucial cells to prime

naïve T cells to effector cells. Differentiation of monocytes here may be started by activated monocytes priming naïve T cells and primed T cells could drive the monocyte differentiation to dendritic cells. Beyond its importance for the CAPRI procedure, the discovery of monocytes as central cells passing over tumor-immunogenic information to T cells could be exploited not only for cellular therapies. Cytokine regimens could perhaps be used to stimulate and differentiate monocytes *in vivo* to prime naïve T cells to tumor-specific effector T cells.

The role of dendritic cells in the priming phase was surprisingly less dominant than that of monocytes, but their depletion at any step of the cascade procedure lessened cancer cell destruction approximately by half. It is not known whether dendritic cells or subsets of dendritic cells differ from monocytes in the type of material they capture and process. Alternatively, uptake of tumorimmunogenic material could silence only subsets of dendritic cells, but their ability to present may be restored upon appropriate activation. It was recently reported that signals from activated $CD4^+$ T cells enable dendritic cells to instruct bystander dendritic cells to prime naïve $CD4^+$ T cells[41,42]. However, in the CAPRI procedure CD3-acivated T cells could not initiate this dendritic circuit because priming of naïve/resting T cells could not be noticed. Our present study shows that depletion of $CD14^+$ monocytes from CAPRI cells immediately before coculture with cancer cells did not significantly reduce cancer cell destruction (**Fig. 5a,b**) . Depletion of dendritic cells before coculture of CAPRI cells with cancer cells reduced cancer cell destruction by half. (**Fig. 5a,b**). This suggests that dendritic cells could provide a continuous flow of cytokines and/or of tumor-immunogenic information during the phase of cancer cell destruction by building a bridge of information between cancer cells and the effector T cells. Thus the activity of tumor-specific T effector cells could be maintained. This may be important particularly for cancer cells which show a low MHC expression. Supplementary

professional presentation by activated dendritic cells apparently prevents rudimentary TCR signalling by cancer cells leading, for example, to default secretion of suppressive IL-10 by Th1 cells[43]. In summary, depletion experiments showed that an optimal priming for optimal cancer destruction required cell-mediated bi-directional cooperations between a cellular quartett consisting of $CD14^+$ monocytes, $CD14^-$ $CD1a^+CD83^+$ dendritic cells, $CD4^+$ and $CD8^+$ T cells.

CAPRI cells lysed a series of different autologous cancer cells (listed in **Table 1**, lysis not shown). Of particular note was the successful lysis of carcinoma in situ cells of Bowens disease by CAPRI cells. These intraepidermally growing carcinoma in situ cells are commonly recalcitrant to therapy because they are enveloped by fibroblasts. Less than one percent of the cells of the Bowen cancer cell line bound keratinocyte antibodies in cytospins (not shown). This in situ cancer is an excellent example for the proposed inhibitory role of tumor stroma as this stroma usually prevents straightforward lysis by T cells[44]. We interpreted the lysis of Bowens in situ cancer cells by CAPRI cells as evidence for crosspresentation and crosspriming between members of the CAPRI cell quartett and perhaps as evidence for the ability of CAPRI cells to enhance fibroblast processing and presentation of tumor products, as has been described[45].

The observed reduction of $CD4^+CD25^{high}$ $FOXp3^+$ regulatory cells during the cascade priming (**Supplementary Fig. 1** online) fits present interpretations of $CD4^+CD25^{high}$ $FOXp3^+$ regulatory cells as suppressing anti-tumor activity. Hence, reduction of $CD4^+$ $CD25^{high}FOXp3^+$ cells should allow cytolytic activity of CAPRI cells. Whereas CD3-stimulation of T lymphocytes favours pathways leading to $CD25^{high}Foxp3^+$ expression[36], the natural activation via the $\alpha\beta$ TCR[37] may favour amplification of $CD4^+$T cells, which do not express FOXp3.

The first case-controlled study with CD3-activated PBMC showed a small but significant reduction of recurrence and an increased survival rate in patients with hepatocellular carcinoma[46]. This result was interpreted as amplification of a small number of cancer specific T memory cells and not as result of CD3-activation of naïve T lymphocytes[47]. This interpretation is compatible with our results of a marginal lysis of cancer cells by CD3-activated PBMC.

Adoptive immune cell therapies have often employed only one effector T cell type or one APC cell type to elicit an immune response against only one tumor-immunogenic peptide. These strategies should be reconsidered, since each population of the CAPRI cell quartett was pivotal in the functional circuits of priming or cancer cell lysis. Consequently, engineering peptides for helper and cytotoxic T cell responses would require design of tumor-immunogenic peptides which would be presented by HLA class I and HLA class II molecules. CAPRI cells increased/induced HLA class I and HLA class II expression in cancer cells and efficiently lysed different types of cancer (listed in **Table 1**). CAPRI-activated APC presenting tumor-immunogenic peptides could be isolated from the peripheral blood of cancer patients as suggested for immunogenic peptides from HIV resistant individuals[48].

Clinical evidence of the CAPRI cell concept was obtained in human patients and in mice. A breast cancer tumor as well as a colorectal tumors were established in twenty-four nude mice. The survival time of CAPRI cell treated mice (N=12) was significantly increased compared to PBMC treated control mice ($P< 0.001$, Fig, supplementary tables...on line). In human patients, CAPRI cell-treated breast cancer patients (N=42) with metastasis showed in an intention-to-treat setting a highly significant improvement of survival rates after five years compared to breast cancer patients with metastasis of the Munich tumor center (Fig, $p=0.0001$). Most patients of the Munich tumor center and most patients with adjuvant CAPRI cell therapy

received the standard combination of chemotherapy and radiation. It is unclear whether the standard therapy hindered or supported the CAPRI cell therapy. Damage of precursor immune cells in the bone marrow would account for adverse effects and could be avoided by leukapheresis before standard procedures. Huge tumor masses prevented from growing by standard procedures would support CAPRI cell therapy in these patients, if PBMC would be isolated before their damage by chemotherapeutic modalities.

Our discovery that monocytes are a goldmine of tumor-immunogenic information can be exploited in many ways. Tumor-immunogenic peptides must not be identified to initiate adoptive cell therapy. Adoptive cell therapy can be started immediately before resection of a tumor at any stage of the cancer development. This would prevent growth of often unknown residual cancer cells, which profit from growth factors produced after surgery for the healing process. Furthermore, lack of negative side effects allows a continuous prophylactic therapy in fast growing/recurring cancer types. We consider the efficient lysis by CAPRI cells of many different types of autologous cancer cells groundbreaking because it demonstrates the immunogenicity of many cancer types as well as a successful method of treatment.

Materials and methods

Tumor samples and establishment of autologous tumor cell lines

Immune cells and autologous tumor samples were donated by informed and consenting patients referred by doctors for support of radiation- or chemotherapy with adjuvant adoptive immunotherapy. Tumor samples were used to establish cancer cell lines to control lytic capacity of activated immune cells. The ethic recommendations of Helsinki with subsequent amendments of Tokyo 1975, Hong Kong 1989 and Somerset West 1996 were followed.

Tumor samples were minced to small pieces and cultured in 50ml-flasks using supplemented tumor culture medium (RPMI 1640 with L-glutamine, supplemented with 10% FCS, NEAA, G5 Supplement, all from PAA, Coelbe, Germany) and optimized culture conditions, unused tumor samples were also minced to small pieces and cryopreserved in DMSO like PBMC[49]. Establishment of cell lines with at least twenty cell divisions was successful only with samples from patients before chemo- or radiation therapy. The following cancer cell lines were used in most experiments: colon cancer line CRC5 was established from a patient´s liver metastasis, the melanoma cell line MEL12 from a recurring tumor (**Table 1**). Nine breast carcinoma lines as well as a bowenoid cell line were established from patients from southern Germany (**Table 1**). All cell lines originated from Caucasian patients.

Isolation of immune cells

PBMC were isolated from venous blood puncture or leukapheresis samples by density gradient centrifugation as described elsewhere[49] using lymphocyte separation medium (LSM, PAA, Coelbe, Germany). Immune cells were either used immediately or cryopreserved and stored in the nitrogen gas phase. Isolation, cryopreservation and thawing procedures as well as the use of optimized culture conditions (38.5°C, 6.5% CO2) were described in detail[49].

Activation of T cells in PBMC bulk cultures: CD3-activation and CAPRI cell generation

Both methods started with activation of T cells in PBMC bulk cultures using the CD3 monoclonal antibody OKT3 (Orthoclone, Cilag, Sulzbach/Taunus, Germany) binding

to the non-polymorphic ε-chain of the CD3 molecule and addition of Interleukin 2 (IL-2; Proleukin, Chiron, Ratingen, Germany).

CD3 antibodies were immobilized at a concentration of 1µg/ml, in 0.05M borate buffer pH 8.6 and distributed in 50ml tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). Coated flasks were kept at 4°C (at least over night) and washed twice with phosphate buffered saline prior to incubation with PBMC. PBMC were added at a concentration of $2x10^6$ per ml in a total volume of 10ml, IL-2 was added within 2-12 hours at a concentration of 20U/ml. CD3-activated cells were expanded on day 4 with IL-2 (20U/ml) and harvested on day 7 for immediate use or cryopreservation.

For the generation of cascade primed (CAPRI cells) CD3-activated PBMC were removed from the flask after 4-6-hours, washed and cocultured in a second CD3 "antibody free" flask with an equal number of unstimulated autologous PBMC (at a concentration of $2x10^6$ per ml in a total volume of 10ml), which contained naïve/resting T cells. Cells were expanded on day 1 with IL-2 (20U/ml) and harvested on day 4.

Flow cytometry

Expression of cell surface markers was determined by flow cytometry using the Becton-Dickinson FACScan analyser and CellQuest software (Becton-Dickinson, Germany). $CD14^+$ cells were CFSE-labelled to trace changes in phenotype. In brief, cells were harvested and stained with anti-CD14 PE, HLA-DR-PE, CD1a-PECy5.5, with anti-CD40-PECy5.5, CD80-PECy5.5, CD83-PECy5, CD86-PECy5.5 and with anti-HLA-A,B,C-PECy5.5 for tracing the phenotype of CFSE labeled CD14 cells during CD3 or CAPRI stimulation. For acquisition of cell surface markers of CD3-stimulated and CAPRI cells, cells were collected and stained with anti-CD3-FITC,

CD14-PE, CD19-PECy5.5 and with anti-CD3-FITC, CD4-PE, CD8-PECy5.5 and with anti-CD3-FITC, CD14-PE, CD56-PECy5.5 and with anti-CD3-FITC, CD16-PE, CD56-PECy5.5. For Foxp3 staining, cells were stained first with anti-CD4-PE, fixed, permeabilized with human Foxp3 staining buffer set and stained with FITC-anti-human Foxp3.

The conjugated mouse monoclonal antibodies were obtained from BD Biosciences or eBioscience. Human Foxp3 staining buffer set was obtained from eBioscience.

Separation of PBMC subpopulations with magnetic beads

Mouse anti–human CD3, CD4, CD8, CD14 conjugated to magnetic beads, CD14 negative isolation kits and Pan mouse IgG beads were obtained from Dynal (Invitrogen, Paisley, UK) and used according to manufacturer's instructions.

$CD4^+$ T cells were isolated from CD3 isolated populations to spare $CD14^+CD4^+$ monocytes.

Microscopic classification, preparation of tumor target cells and quantification of cancer cell destruction using the Cr^{51} release assay

Cancer cells were removed from flasks by trypsinization, resuspended in 10% FCS enriched culture medium (RPMI 1640 with L-Glutamine, PAA, Coelbe, Germany) and washed twice. Cancer cells were counted and distributed in different concentrations in 96-well flat bottomed culture plates (Falcon, Becton Dickinson, Heidelberg, Germany) either for microscopic evaluation of lysis or for the Cr^{51} release assay. Cancer cells were allowed to recover over night to restore their membranes after trypsinization before addition of effector cells. Reorganization of cancer cells into a coherent cell layer better reflected the situation *in vivo* and significantly lowered the rate of spontaneous release of Cr^{51}, a so far unresolved problem of the Cr^{51} release assay in measuring tumor cell destruction.

The Cr^{51} release assay was performed in duplicates at varying effector : target ratios, using 2 x 10^3 cancer cells as targets. Maximum release was determined using labelled target cells, spontaneous release by incubating target cells in medium alone. Percent spontaneous release was calculated as follows: (spontaneous cpm : maximum cpm) x 100; and the percent cytotoxicity was calculated as follows: [(experimental cpm – spontaneous cpm) : (maximum cpm – sponatanious cpm)] x 100. Quantitative lysis of cancer cells using the Cr^{51} release test was assessed after 5-6h and after 18-22h, following the "classical" guidelines of the CML assay[23] with the crucial difference of tumor target preparation described above.

Estimation of the degree of lysis was performed by microscopic inspection after 18-24h. The scale used corresponded to conventional HLA microscopic estimated evaluation, designating more than 80% cell lysis as strong positive (++) and 60-79% as positive; 40-59% as weak positive, 20-39% (+) as doubtful positive and less than 20% (-) as negative (**Fig 2**).

To determine the influence of HLA class I and class II molecules on cancer lysis, monoclonal antibodies were added at the start of the immune cell - cancer cell cocultures. The antibody W6/32 (abcam, Cambridge, UK) was used to block HLA class I (1μg/ml) and HLA workshop-defined anti DQ2 antibody C1A2 (1μg/ml) (11WO938; WAN 806, kindly provided by Prof. J.P. Johnson, Munich) was used to block HLA class II.

Patient panel, CAPRI cell dose and schedule of treatment

All breast cancer patients with diagnosed metastasis (N= 42), who received at least 500 x 10^6 CAPRI cells (although higher cell amounts were recommended and often received), were included in the analysis and compared to breast cancer patients with the same tumor staging of the Munich Tumor Center (N=). Inclusion was independent from the type of chemotherapy and/or radiation or other therapies. The

prefered schedule of treatment was three times/week with 60 - 80 x 10^6 CAPRI cells for 6 months, continued by two injections every week for another 6 months. ACT with CAPRI cells has been continued in most of the patients one time weekly for several years. One third of CAPRI cells were injected i.v., two thirds i.c and i.m. in a volume of 1ml PBS in the forearm.

The melanoma patient (female, 67years of age, first diagnosis x years before entering CAPRI cell therapy, several op.....) was injected x CAPRI cells twice weekly at the border of the melanoma. No other therapeutic modalities were performed 6?months before and during the CAPRI cell therapy.

Statistical analysis

The slope and y intercept of regression lines obtained from CML titrations were evaluated using the General Linear Model (GLM) procedure. The statistical package SPSS 10.1 (SPSS Inc., Chicago, Illinois) was used.

Kaplan.....

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Authors contribution

Barbara Laumbacher and Rudolf Wank pioneered the CAPRI cell procedure over several years. Songhai Gu designed and performed the elegant FACS experiments. All authors participated in writing the manuscript.

Competing interests statement

Barbara Laumbacher and Songhai Gu have no conflicting interests.

Rudolf Wank holds European and International patents for the CAPRI procedure,

Legends to figures

Figure 1

Comparison of cancer cell lysis by CAPRI cells and by CD3-activated PBMC. Breast cancer cells (**a,b**), CD3-activated PBMC (**c**) and CAPRI cells (**d**) were subsequently cocultured (**e,f**). The degree of lysis by CD3-activated PBMC (**e**) or CAPRI cells (**f**) was compared microscopically (**c,d**) and by the chromium^{51} release test (**g**) after 20h. Effector (E) to target (T) ratio was 20:1 (**a,b,c,d,e,f,g**) titrated to 5:1 in the chromium^{51} release assay (**g**). The number of cancer cells appeared unchanged with cocultured CD3-activated PBMC after 20h (**e**), whereas CAPRI cells clearly reduced the number of cancer cells (**f**) as demonstrated quantitatively using Cr^{51} labelled cancer cells as targets (**g**).

Our modification of the CML (cell mediated lympholysis) test[23] kept the spontaneous release under 10% during assay periods of 18-24h.

Figure 2

CAPRI cells lyse cancer cells MHC restricted. MHC restricted lysis by CAPRI cells, using a E:T ratio of 20:1, was determined microscopically after 24h in two ways: (**a**) by using autologous cancer cells and cancer cells sharing or not sharing HLA-DQ alleles, (**b-f**) by presenting lysis and HLA-blocked lysis of autologous cancer cells. (**a**) autologous cancer cells were lysed the strongest (++); cancer cells sharing HLA-DQB1*0603 or DQB1*0201 with CAPRI-CTL1 (upper line, HLA-DQB1*0201 and DQB1*0202 express identical surface molecules), or sharing HLA-DQB1*0604 or DQB1*0301 with CAPRI-CTL2 were noticeably lysed (+). No significant lysis occurred without HLA-DQ class II matching; the quantity of lysed cancer cells was estimated analogous to the HLA microcytotoxicity test. (**b**) autologous breast cancer

cells without CAPRI cells, (**c**) lysis of autologous cancer by CAPRI cells, (**d**) lysis blocked by HLA-DQ class II antibody, (**e**) lysis blocked by HLA class I antibody, (**f**) no blocking of lysis by isotype control antibody.

Figure 3

Increased HLA class I and HLA class II expression of breast cancer cells after cocultivation with CAPRI cells. To save sufficient unlysed cancer cells for observation a low effector:target (E:T) ratio of 5:1 was used. HLA expression of CFSE-labelled colon cancer cells F2 was determined after five days of coculture with (**a**) CAPRI cells, (**b**) CD3-activated PBMC or (**c**) unstimulated PBMC. CAPRI cells stimulated cancer cells to a mean increased fluorescence (MIF) expression of HLA class I by 40% and of HLA-DR class II antigens by 60% (**a**). No significant change of HLA expression of cancer cells could be observed after incubation with CD3-activated (**b**) or unstimulated PBMC (**c**).

Figure 4

Expression of differentiation and activation markers by CFSE labelled $CD14^+$ monocytes during cascade priming and CD3-activation of PBMC. PBMC with reimported CFSE-labelled $CD14^+$ monocytes were split in three groups: PBMC were left unstimulated (**a**), submitted to cascade priming (**b**) or CD3-activated (**c**). Developmental and functional stages of labelled monocytes were characterized with specific antibodies recognizing CD14 (monocytes), CD83 and CD1a (dendritic cells), CD80, CD86, CD40 (activation markers) and HLA-DR class II and HLA class I molecules at days 0 (**a**), 1 (**b,c**) and 5 (**b,c**). Whereas the number of $CD14^+$ monocytes declined in cascade primed cells cultures at days 1 and stronger at day 5 (**b**), numbers of $CD14^+$ monocytes in unstimulated (**a**) and in CD3-activated PBMC

(**c**) remained essentially unchanged at days 1 and 5. In contrast, numbers of $CD83^+$ and $CD1a^+$ cells increased in cascade primed cell cultures (**b**) but not in CD3-activated PBMC and unstimulated PBMC (**a,c**). Increased expression of activation markers CD80, CD86, CD40 and HLA-DR class II and HLA class I molecules was clearly stronger in cascade primed cell cultures (**b**) than in CD3-activated PBMC and unstimulated PBMC (**a,c**).

Figure 5

Interdependence of monocytes, dendritic cells, T helper and T cytotoxic cells for priming and cancer cell lysis.

The contribution of immune cell populations to the cytotoxic capacity of CAPRI cells against breast cancer cells was determined at different phases of activation and cancer cell destruction (**a**) by depleting PBMC from $CD14^+$ monocytes, (**b**) from $CD83^+$ dendritic cells, (**c**) from $CD3^+CD4^+$ T helper cells, or (**d**) from $CD3^+CD8^+$ T cytoxic cells. Depletion occurred at the following points in time: before CD3-activation of PBMC (···◇···), from unstimulated PBMC to be added to CD3-activated PBMC for T cell priming (···△···), from CAPRI cells before incubation with cancer cells (···●···). Not depleted CAPRI cells (—■—) ensured validity of tests (**a,b,c,d**). (**a**) Presence of $CD14^+$ monocytes was absolutely required for development of cytotoxicity in the priming procedure, during CD3 activation of PBMC and during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 0.003$, $P_{intercept} = 0.001$, for both PBMC populations) and had minimal non-significant impact on the lytic power of CAPRI cells ($P_{slope} = 0.37$, $P_{intercept} = 0.057$). (**b**) Depletion of $CD83^+$ dendritic cells reduced development of cytotoxicity during CD3-activation of PBMC ($P_{slope} = 0.00072$, $P_{intercept} = 0.008$), during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 0.004$, $P_{intercept} = 0.007$) and reduced also the lytic power of CAPRI cells significantly ($P_{slope} = 0.01$, $P_{intercept} =$

0.004*)*. There was no significant difference between the dendritic cell depletions at the three points of time. (**c**) $CD3^+$ $CD4^+$ T helper cells were absolutely required during the CD3-activation of PBMC ($P_{slope} = 1.25 \times 10^{-7}$, $P_{intercept} = 0.022$), during priming of PBMC with CD3-acivated PBMC ($P_{slope} = 1.59 \times 10^{-6}$, $P_{intercept} = 0.015$) and for cytotoxicity of CAPRI cells ($P_{slope} = 1.59 \times 10^{-6}$, $P_{intercept} = 0.015$) . (**d**) $CD8^+$ T cytoxic cells had a key role in all phases of the cascade priming procedure and cancer cell lysis ($P_{slope} = 0.003$, $P_{intercept} = 0.001$, at all three points of time).

EXHIBIT B

i n v i v o killing test

1-1 Nude mice and cells

1-1-1 Nude mice

For testing the Capri cell *in vivo* killing, nude female mice, which were 6 weeks old, were obtained from CDCC, Wuhan, Wuhan University. Aseptic procedures were used as usual. Each group included 6 mice.

1-1-2 Cells

Tumor cell lines from a patient with breast cancer (ZB) and a patient with colorectal cancer (FW) were established as described. PBMC were obtained from both patients. Unstimulated PBMC were used only in control groups.

CAPRI cells

1-2 Method

1-2-1 Schedule of injection:

	ZB		FW	
	Control group	TEST Group	Control group	TEST Group
Day 0	ZB tumor cells + PBMC	ZB tumor cells + CAPRI	FW tumor cells +PBMC	FW tumor cells +CAPRI
Day 1	PBMC	CAPRI	PBMC	CAPRI
Day 3	PBMC	CAPRI	PBMC	CAPRI
Day 5	PBMC	CAPRI	PBMC	CAPRI
Day 7	PBMC	CAPRI	PBMC	CAPRI
Day 9	PBMC	CAPRI	PBMC	CAPRI
Day 11	PBMC	CAPRI	PBMC	CAPRI
Day 13	PBMC	CAPRI	PBMC	CAPRI
Day 15	PBMC	CAPRI	PBMC	CAPRI

Tumor cells were thawed and washed twice in PBS, counted and resuspended in PBS, at a concentration of

$1x10^6$ cells/ml. Each mouse was injected subcutaneously 0.1ml tumor cell suspension containing $1x10^5$ tumor cells. PBMC and CAPRI cells were washed twice in PBS, counted and resuspended in PBS at a concentration of $1x10^7$ cells/ml. Each mouse was injected subcutaneously 0.1ml PBMC or Capri cell suspension containing $1x10^6$ cells. The injection sites were around the tumor injection sites.

1-2-2

After the injection of tumor, mice were observed for 45 days. When the maximum diameter was bigger than 15mm, the individual mouse was killed. After 45 days the experiment was finished, all animals were killed.

1-2-3

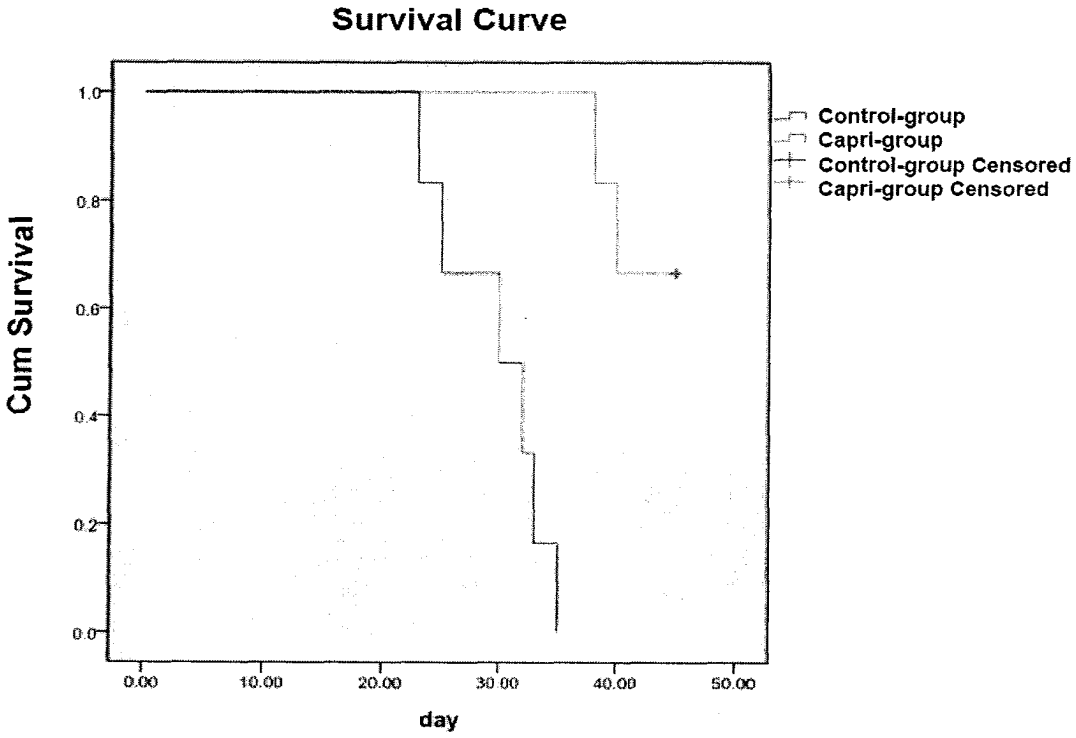
Tumor size was measured at day 21 unless the mouse was killed already by the tumor or was executed when the the tumor maximum diameter was bigger than 15mm.

2 results

2-1 observation of tumor size

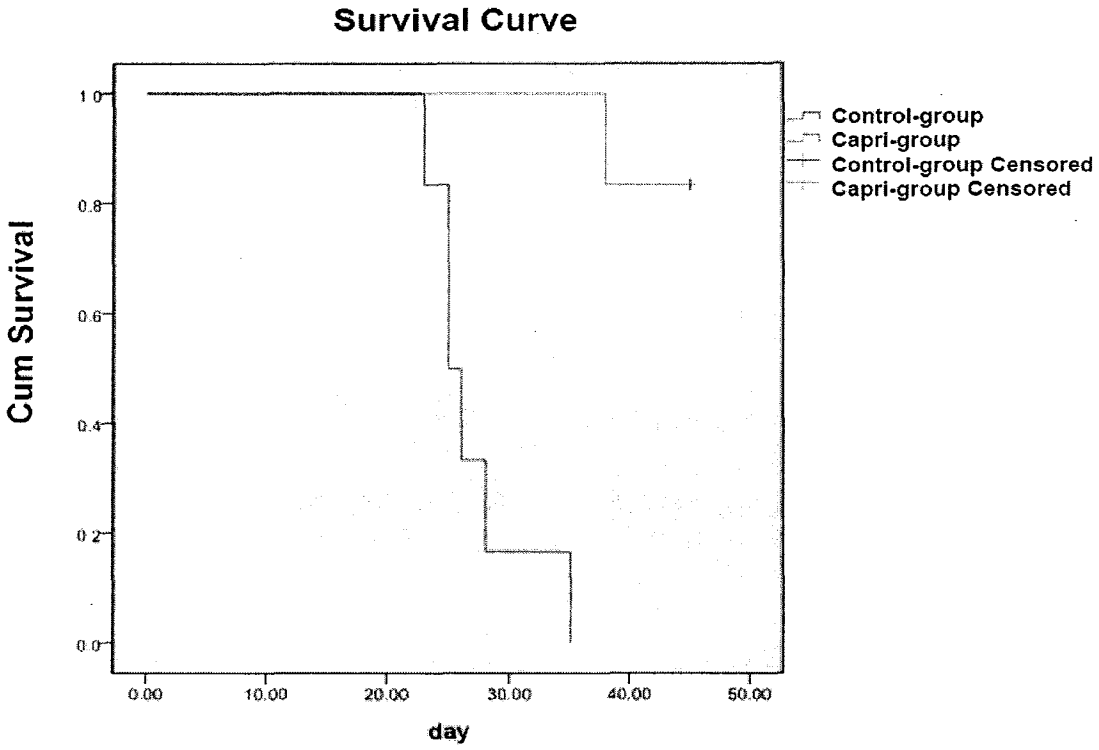
	ZB		FW	
	Control group	TEST group	Control group	TEST group
	ZB tumor cells + PBMC	ZB tumor cells + CAPRI	FW tumor cells + PBMC	FW tumor cells + CAPRI
	Max. ø x Min. ø	Max. ø x Min. ø	Max. ø x Min. ø	Max. ø x Min. ø
Mouse 1	6.21 x 5.82	2.22 x 2.14	5.30 x 4.29	3.01 x 2.87
Mouse 2	5.51 x 5.35	1.98 x 1.45	4.95 x 4.45	2.11 x 2.05
Mouse 3	5.64 x 5.23	2.85 x 2.45	5.54 x 5.48	2.05 x 1.89
Mouse 4	5.54 x 5.39	2.73 x 2.38	6.22 x 5.90	2.26 x 2.16
Mouse 5	4.21 x 4.11	2.54 x 2.33	5.21 x 4.88	2.29 x 2.65
Mouse 6	6.54 x 5.58	1.99 x 1.75	6.21 x 5.89	3.01 x 2.97
Average tumor size (mm^2)	29.64±6.95	5.08±1.66	28.95±6.60	5.78±2.70
T test	**t=8.41, P=7.56X10^{-6}**		**t=7.96, P=1.23X10^{-5}**	

CAPRI cells reduced the tumor size significantly in both tumor models.



2-2 Survival curve

In the breast cancer model, Kaplan-Meier test showed that the average survival time in the TEST group was 43.00±1.17days, while the average survival time in the control group was 29.67±1.92 days, **p<0.001**.



In the colorectal cancer model, Kaplan-Meier test showed that the average survival time in the TEST group was 43.83±1.07 days, while the average survival time in the control group was 27.00±1.73 days, **p<0.001.**

EXHIBIT C

ADOPTIVE CELL THERAPY OF AUTOLOGOUS CANCER WITH AUTOLOGOUS IMMUNE CELLS FROM THE PERIPHERAL BLOOD (PBMC)

INVENTOR	ACTIVATION 1	ACTIVATION 2	LYSIS OF AUTOLOGOUS CANCER	LYSIS OF ALLOGENEIC CANCER
Babitt et al.	CD3 antibody + PBMC	T3CS + CD3 + PBMC	**NO** or very weak	**YES** strong K562
Gold et al.	CD3 antibody	no activation 2	unreliable, dependent on memory T cells	**YES** strong
Wank	CD3 antibody	activated APC-PBMC + PBMC with naïve T cells	**YES** strong	**NO** lowgrade if HLA antigens are shared

CD3 activations with the methods of Babitt et al. and Gold et al. cause an increased frequency of FoxP3 suppressor cells. Therefore CD3 antibodies are today used to prevent rejection of transplants. They suppress the activation via the $\alpha\beta$ TCR. The method of Gold et al. may increase the number of few T memory cells against cancer but causes a considerable increase of FoxP3 suppressor cells.
The equation of activation via CD3 with an activation via the $\alpha\beta$ TCR was an error shared by many investigators at that time. However, it was unexspected that activated antigen presenting cells (APC) would contain specific processed tumor antigens (peptides), and that this information could be transferred and be immunogenic to naïve T cells.